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REVIEW

The chemistry and enzymology of the type I signal peptidases

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Abstract

The discovery that proteins exported from the cytoplasm are typically synthesized as larger precursors with cleavable signal peptides has focused interest on the peptidases that remove the signal peptides. Here, we review the membrane-bound peptidases dedicated to the processing of protein precursors that are found in the plasma membrane of prokaryotes and the endoplasmic reticulum, the mitochondrial inner membrane, and the chloroplast thylakoidal membrane of eukaryotes. These peptidases are termed type I signal (or leader) peptidases. They share the unusual feature of being resistant to the general inhibitors of the four well-characterized peptidase classes. The eukaryotic and prokaryotic signal peptidases appear to belong to a single peptidase family. This review emphasizes the evolutionary concepts, current knowledge of the catalytic mechanism, and substrate specificity requirements of the signal peptidases.

Keywords: endoplasmic reticulum; leader peptidase; membrane protein; protein secretion; signal peptidase

Over two decades ago, Milstein and colleagues (1972) reported the existence of peptidases that are involved in the maturation of secreted proteins. Their key observation was that polysomes from myeloma cells synthesized an immunoglobulin light chain with a higher molecular weight than that of the secreted protein and it was the microsomes, not the microsomally-derived polysomes, that produced a light chain with the mature molecular weight. Soon it was found that a large number of secreted and membrane proteins are synthesized in a higher molecular weight form with an extra amino-terminal signal (or leader) peptide and that these signal peptides are essential for export from the cytoplasm (Carlson & Botstein, 1982; Michaelis & Beckwith, 1982). These signals share some common features (von Heijne, 1983): They are basic at the N-terminus, apolar in the middle, and have small, uncharged amino acid residues preceding the site of cleavage by the signal (or leader) peptidase. Genetic studies (Emr et al., 1978; Bassford & Beckwith, 1979; Bedouelle et al., 1980; Emr & Silhavy, 1980) have revealed that the integrity of the hydrophobic stretch of signal peptides is vital for protein transport across the membrane barrier.

Progress toward characterization and purification of type I signal peptidases was advanced by the development of in vitro post-translational cleavage assays using precursors of secretory proteins synthesized by cell-free protein synthesis as peptidase substrates. Detergent-solubilized membrane preparations containing signal peptidase were found to correctly cleave the signal peptides from those full-length precursor proteins (Szczesna & Boime, 1976; Jackson & Blobel, 1977; Kaschnitz & Kreil, 1978; Jackson, 1983; Lively & Walsh, 1983). The addition of phospholipids was necessary to maintain the activity of signal peptidase in detergent-solubilized membranes (Jackson & White, 1981). The microsomal signal peptidase was first purified from canine pancreatic microsomes (Evans et al., 1986) and subsequently from endoplasmic reticulum (ER) microsomes isolated from the magnum region of the oviducts of laying hens (Baker & Lively, 1987).

The first prokaryotic signal (leader) peptidase was isolated from *Escherichia coli* by Zwizinski and Wickner (1980) using an assay that monitored the conversion of the precursor form of the M13 coat protein, termed procoat, to the mature coat protein. A year later, the *lepB* gene encoding this signal peptidase was cloned (Date & Wickner, 1981) and subsequently sequenced (Wolfe et al., 1983). The signal peptidase encoded by *lepB* was purified from a strain which overproduces the protein (Wolfe et al., 1982) thus permitting further characterization of its enzymatic properties.

Initial comparisons of the activities of the bacterial and eukaryotic signal peptidases revealed that the substrate specificities of each were very similar. M13 procoat, a prokaryotic precursor pro-

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tein, was correctly cleaved by a detergent-solubilized extract of canine pancreas microsomes (Watts et al., 1983). Conversely, the insulin precursor protein, a eukaryotic substrate, was cleaved in vivo at the correct processing site by the bacterial signal peptidase (Talmadge et al., 1980). Thus, early enzymatic data suggested that the prokaryotic and eukaryotic enzymes are possibly related enzymes.

Early attempts to define the mechanistic class of the signal peptidases were unsuccessful because the processing activities of the *E. coli* peptidase (Zwizinski et al., 1981) and the canine pancreas ER peptidase (Jackson & Blobel, 1980) were insensitive to the usual reagents known to be general inhibitors of the four well-characterized peptidase classes. The mechanism of action of signal peptidases remains undefined even today but significant progress has been made with the isolation and characterization of the proteins and the genes that encode them. Recent studies suggest that signal peptidase may use a novel serine peptidase mechanism. Here we provide an overview of the current state of our knowledge of signal peptidase structure and function. For a recent review of individual signal peptidases, the reader may consult the book by von Heijne (1994).

The physiological role of processing

Signal peptidase is an essential enzyme in *E. coli* (Date, 1983), in the yeast *Saccharomyces cerevisiae* (Böhni et al., 1988) and presumably in most other cells. The cleavage of the signal peptide is not required for translocation of precursor proteins through the membrane as pre-proteins that have defective cleavage sites may be found translocated completely across the membrane (Koshland et al., 1982; Kuhn & Wickner, 1985; Fikes & Bassford, 1987). However, these precursors remain membrane-bound because the uncleaved hydrophobic signal peptide acts as a membrane anchor. Furthermore, using a conditional-lethal mutant of *E. coli* in which the expression of the *lep* gene was under the control of the arabinose promoter, it was shown that uncleaved precursors are also translocated across the membrane in the absence of signal peptidase and remain anchored to the periplasmic side of inner membrane (Dalbey & Wickner, 1985). Similarly, a mutant form of human coagulation factor X with a defective signal peptide that blocks cleavage by signal peptidase is translocated into the ER where it remains as a membrane-bound protein that does not progress further along the secretory path for export from the cell (Racchi et al., 1993). Finally, some purified precursor proteins are active without removal of the signal peptide (Haugen & Heath, 1979; Ito, 1982), indicating that cleavage is not always required for the correct folding and activity of the exported protein. Thus, the role of signal peptidase in vivo is to release exported proteins from the membrane so they can reach their correct cellular or extracellular locations.

An intriguing result was very recently obtained that suggests that some cells may function without a signal peptidase or that there are other, currently unrecognized types of signal peptidase. The determination of the entire DNA sequence of the genome of the bacterium *Mycoplasma genitalium* (Fraser et al., 1995), which is thought to have the smallest genome of all known self-replicating organisms, revealed that this organism lacks a gene sequence with any recognizable similarity to the type I signal peptidase family. Despite this, the genome sequence contains eleven putative precursor proteins with type I signal peptidase cleavage sites. It is not known whether these proteins are secreted normally without cleavage or whether an unrecognized gene encodes a different signal peptidase.

Subunit composition and membrane topologies of signal peptidases

Eubacterial signal (leader) peptidase

The best known type I signal peptidase is that of *E. coli*. It consists of a single 37 kDa polypeptide chain (Wolfe et al., 1982) and is an integral membrane protein in the plasma membrane. The protein spans the membrane twice (Fig. 1B) with a small N-terminal end and a large carboxyl-terminal domain located in the periplasmic space (Wolfe et al., 1983; Moore & Miura, 1987; Whitley et al., 1993). The active site of this enzyme is located in the periplasm (Bilgin et al., 1990).

Genes encoding signal peptidase have been isolated from other bacteria as well. The signal peptidases from the Gram negative bacteria *Salmonella typhimurium* (van Dijl et al., 1990), *Pseudomonas fluorescens* (Black et al., 1992), *Haemophilus influenzae* (Fleischmann et al., 1995), *Rhodobacter capsulatus* (GenBank ac-

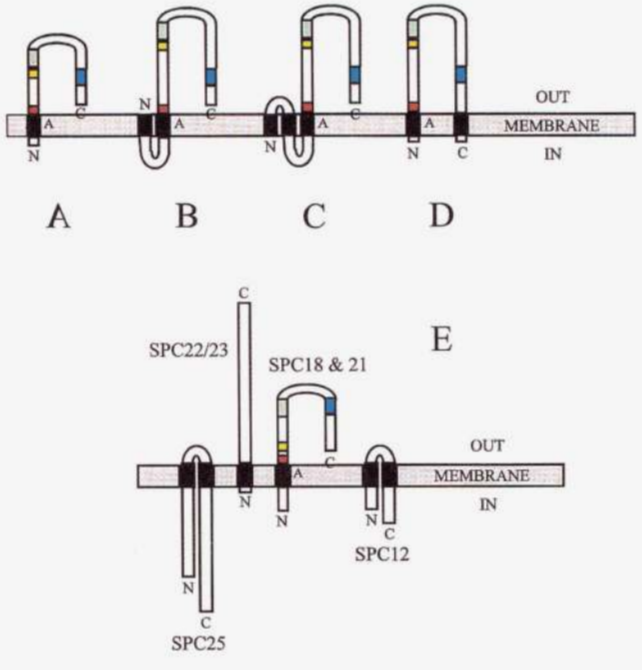

Fig. 1. Membrane topology of bacterial, mitochondrial and ER signal peptidases. Predicted membrane topologies are shown for signal peptidases of **(A)** *B. japonicum*, *P. laminosum*, *M. tuberculosis*, *S. aureus*, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. caldolyticus*, and the inner membrane of yeast mitochondria (Imp1p); **(B)** *E. coli*, *S. typhimurium*, and *P. fluorescens*; **(C)** *H. influenzae*; **(D)** *R. capsulatus*, and the inner membrane of yeast mitochondria (Imp2p); and **(E)** the canine ER membrane (SPC12, SPC18, SPC21, SPC22/23, and SPC25). "IN" indicates the cytoplasmic side of the membrane in all models. In models A through D, "OUT" indicates the extracytoplasmic face of the membrane or the intermembrane space in the cases of mitochondrial inner membrane peptidases. In model E, "OUT" represents the lumen of the ER. In each model, the letter "A" within the bilayer indicates the position of the conserved domain A, the letter "N" represents the N-terminus and the letter "C" represents the C-terminus of the proteins. The positions of other conserved domains are indicated in color: black, predicted membrane anchor; red, domain B, containing the putative active site serine residue; yellow, domain C; green, domain D; blue, domain E. SPC, signal peptidase complex.

cession #Z68305), and *Bradyrhizobium japonicum* (Müller et al., 1995) appear to consist of 36, 32, 40, 29, and 28 kDa polypeptide chains, respectively. Based on the alignment of the primary sequences of these signal peptidases with that of the *E. coli* enzyme, topological models with two membrane-spanning regions have been proposed for the signal peptidases of *S. typhimurium* and *P. fluorescens* (Fig. 1B). In contrast, the signal peptidase of *H. influenzae* may possess three amino-terminal membrane-spanning regions (Fig. 1C), and *R. capsulatus* signal peptidase may have one amino terminal and one carboxyl terminal membrane spanning region (Fig. 1D). The signal peptidase of *B. japonicum* appears to have only one membrane-spanning region, which corresponds to the second found in the *E. coli*, *S. typhimurium*, and *P. fluorescens* signal peptidases (Fig. 1A). The latter feature is shared by the signal peptidase of the cyanobacterium *Phormidium laminosum* (22 kDa; Packer et al., 1995), and all known signal peptidases from Gram positive bacteria, which include enzymes from *Bacillus subtilis* (van Dijl et al., 1992), *Bacillus amyloliquefaciens*, *Bacillus caldolyticus*, and *Bacillus licheniformis* (for a recent compilation of sequences see Meijer et al., 1995), *Staphylococcus aureus* (SpsB; Cregg et al., 1996), and *Mycobacterium tuberculosis* (Philipp et al., 1996). All *Bacillus* signal peptidases and the *S. aureus* signal peptidase consist of 21 kDa polypeptides whereas the *M. tuberculosis* gene encodes a 32 kDa polypeptide.

Interestingly, both in *B. subtilis* and *B. amyloliquefaciens*, chromosomal genes were identified for two homologous, but non-identical, type I signal peptidases, denoted SipS and SipT (van Dijl et al., 1992; Meijer et al., 1995; Hoang & Hofemeister 1995; GenBank accession #U45883). In addition, a third chromosomal gene for a homologous type I signal peptidase (SipU) was identified in *B. subtilis* (Akagawa et al., 1995; H. Tjalsma, S. Bron & J. M. van Dijl, unpubl. obs.). Of these three *B. subtilis* enzymes, SipS is best-studied. SipS is a non-essential enzyme for survival of *B. subtilis* yet it is important for protein secretion because cells lacking SipS secrete several proteins at greatly reduced levels (Bolhuis et al., 1996). Whether this is also true for SipT and SipU is not yet known.

In addition to the chromosomal-encoded *sip* genes, certain strains of *B. subtilis* contain signal peptidase-encoding genes, denoted *sipP*, that are specified by endogenous plasmids (pTA1015 and pTA1040; Meijer et al., 1995). The amino acid sequences of the two known plasmid-encoded signal peptidases of *B. subtilis* are highly similar to the three chromosomal-encoded signal peptidases (72.2% identical and similar residues in a consensus length of 194 residues). At present, it is not known why *B. subtilis* contains so many distinct signal peptidase-encoding genes. This phenomenon may relate to the fact that, under certain conditions, *bacilli* are able to secrete large amounts of protein. Since the expression of the genes for many secreted proteins is temporally controlled, the expression of certain genes encoding signal peptidase could be controlled in the same way for efficient protein export. Indeed, this was recently shown to be the case for the expression of the *sipS* gene. In addition, SipS, SipT and SipU appear to have a preference for different pre-proteins, indicating that they have different, but overlapping, substrate specificities (Bolhuis et al., 1996).

Unlike SipS of *B. subtilis*, the signal peptidase SpsB from *S. aureus* appears to be an essential enzyme. Nevertheless, *S. aureus* contains a gene, spsA, which specifies a protein that is very similar to SpsB (62% identical and similar residues) and the signal peptidases of *B. subtilis*. However, it seems that the SpsA protein does not possess signal peptidase activity (see the section on the catalytic mechanism of signal peptidases). *SpsA* and *spsB* are adjacent genes on the chromosome of *S. aureus* (Cregg et al., 1996).

Microsomal (ER) Signal Peptidase

In contrast to the bacterial signal peptidases, the eukaryotic microsomal signal peptidases of the endoplasmic reticulum (ER) are multimeric membrane protein complexes. The enzyme from canine pancreas ER microsomes, the first eukaryotic signal peptidase to be purified, is composed of five subunits (Evans et al., 1986). The canine signal peptidase complex (SPC) subunits are named SPC25, SPC22/23, SPC21, SPC18, and SPC12, according to their apparent molecular masses observed by SDS PAGE. The chicken oviduct signal peptidase can be isolated as a complex of two polypeptides, 23 and 19 kDa, that are named gp23 and p19 (Baker & Lively, 1987; Lively et al., 1994). Like the canine complex, partially purified microsomal signal peptidase from the yeast *Saccharomyces cerevisiae* is associated with a complex of at least four proteins of 13, 18, 20, and 25 kDa (YaDeau & Blobel, 1989; YaDeau et al., 1991). The 18 kDa yeast protein was first identified genetically as the Sec11 gene product, Sec11p (Böhni et al., 1988).

The SPC21, SPC18, p19, and Sec11p subunits form a family of related proteins that are believed to contain the peptidase active site(s). The SPC18 (Shelness & Blobel, 1990) and SPC21 (Greenburg et al., 1989) subunits are homologous isoforms with amino acid sequences that are 80% identical to each other and each is approximately 47% identical to Sec11p (Greenburg et al., 1989). Sec11p is required for signal peptide processing and is essential for viability in yeast. Chicken p19 is also a closely related member of this family of proteins (S. J. Walker & M. O. Lively, unpubl.). These subunits are integral membrane proteins that span the membrane once such that the bulk of the protein is located in the lumen of the ER (Shelness et al., 1993; see Fig. 1E).

PCR amplification of mRNA sequences and DNA sequence analysis of multiple clones has shown that chickens, humans, rats, and frogs, like dogs, also encode two isoforms of the Sec11p-like subunit (M. O. Lively & S. J. Walker, unpubl. data). It appears that most eukaryotic species have at least two isoforms of this signal peptidase subunit. In contrast, the *S. cerevisiae* genome (Goffeau et al., 1996) contains only a single gene encoding the Sec11p subunit of signal peptidase. Thus it appears that the multiple forms of signal peptidase genes must have arisen later in the evolution of higher eukaryotes.

While currently available evidence supports the hypothesis that the Sec11p-like subunits contain the peptidase active site, the functions of the apparently redundant isoforms of this protein are not known. Each peptidase isoform may be specific for cleavage of different substrates as shown with the mitochondrial inner membrane peptidases Imp1p and Imp2p (Nunnari et al., 1993). Alternatively, the isoforms may be differentially expressed forms of signal peptidase that are specific for different tissues. The Sec11p-like subunits may also play a role in protein degradation in the ER because the Sec11 strain of *S. cerevisiae* has been found to have a defective protein degradation pathway that requires the Sec11p subunit (Mullins et al., 1995).

Interestingly, the level of an mRNA encoding a member of the Sec11p family was found to be increased nearly two-fold in the brains of rats that had been treated with repeated, high doses of ethanol (Signs & Jacquet, 1994). The physiological basis for this increased transcription is unknown but the observation shows that expression of signal peptidase subunits can be induced under some circumstances.

All known eukaryotic signal peptidase complexes contain a glycoprotein subunit. In chickens and dogs, the glycoproteins are 23 kDa subunits, each with a single Asn-linked, mannose-rich carbohydrate chain (Evans et al., 1986 ; Baker & Lively, 1987). DNA sequences encoding signal peptidase glycoproteins have been characterized from dogs (Shelness et al., 1988), chickens (Newsome, et al., 1992), *Caenorhabditis elegans* (GenBank accession #L14331), *S. cerevisiae* (GenBank accession #X94607), and *Schizosaccharomyces pombe* (GenBank accession #Z69728). The predicted amino acid sequences (180 to 185 residues) of these five subunits have 40.1% identical residues and conservative changes in a consensus length of 192 positions. The chicken and dog subunits are 90% identical to each other. There are more than twelve partial cDNA sequences from human sources in the expressed sequence tag section of GenBank that collectively predict a single open reading frame with 90% identical amino acids compared to the canine and chicken 23 kDa glycoprotein sequences.

While the glycoprotein signal peptidase subunit is present in all species examined from yeast to man, its role in the cleavage of signal peptides is currently unknown. Like the Sec11p subunits, this protein is a type II membrane protein with a short, N-terminal cytoplasmic tail and the bulk of the protein in the lumen of the ER (Shelness et al., 1993; see Fig. 1E). Genetic experiments in *S. cerevisiae* have found that the glycoprotein gene is essential for signal peptide cleavage and for viability of the yeast (H. Fang & N. Green, pers. comm.; E. Beasley, pers. comm.). The glycoprotein subunit appears to be required for proteolysis in vitro because dissociation of the 23 kDa glycoprotein from chicken p19 inactivates signal peptidase (Newsome et al., 1992).

A cDNA encoding SPC25 (Greenburg & Blobel, 1994) reveals a 226-residue protein with a region that is highly similar to a 123-residue coding region of a human cDNA (GenBank accession #D14658) and a 178-residue coding sequence in *S. cerevisiae* (GenBank accession #S498805). Dog SPC25 spans the ER membrane twice such that the bulk of the protein is located in the cytoplasm, on the opposite side of the membrane from the glycoprotein and Sec11-like subunits (Kalies & Hartmann, 1996; see Fig. 1E). This membrane orientation led Kalies and Hartmann to suggest that SPC25 is not likely to play a direct role in signal peptidase catalysis. Consistent with their speculation, genetic studies in *S. cerevisiae* conducted by two different groups have now shown that the yeast homologue of the SPC25 signal peptidase subunit is not essential for signal peptidase activity (H. Fang & N. Green, pers. comm.; E. Beasley, pers. comm.). Interestingly, disruption of the yeast gene encoding the SPC25 homologue in *S. cerevisiae* cells containing the sec11 mutation is lethal (Mullins et al., 1996). The role of the SPC25 homologues in signal peptide processing, if any, has not been defined.

The functional role of the smallest signal peptidase subunit is also unknown. Recently, a number of amino acid sequences of peptides obtained from purified canine SPC12 were directly determined and used to clone a cDNA encoding the human homologue of SPC12 (Kalies & Hartmann, 1996). A gene encoding the 12 kDa subunit of the yeast signal peptidase has also been isolated (Fang et al., 1996). Like SPC25, SPC12 spans the ER membrane twice and is primarily located in the cytoplasm (Fig. 1E). Its role in peptidase activity is unknown and the 12 kDa subunit is not essential for activity of the yeast signal peptidase. Nevertheless, its overproduction resulted in increased signal peptidase activity in the mutant Sec11 strain and the protein appears to be important for efficient signal peptidase activity (Fang et al., 1996). Taken together, these results are consistent with the observation that the gp23 and p19 subunits are sufficient for proteolysis by chicken signal peptidase in vitro and suggest that the SCP25 and SPC12 subunits play indirect roles in the processing of secretory proteins.

Archaebacterial signal peptidase

The sequence of the complete genome of the methanogenic archaeon *Methanococcus jannaschii* has revealed a gene encoding a 24 kDa protein that is a putative signal peptidase (Bult et al., 1996; GenBank locus MJU67481). Interestingly, this protein is more similar to the Sec11-like subunits of the ER signal peptidases (40.4% identical residues and conservative changes in a consensus length of 213 residues) than to the eubacterial signal peptidases. This sequence provides a conceptual link between the eubacterial and eukaryotic sequences that strengthens the hypothesis that the type I signal peptidases may have evolved from a common ancestor.

Mitochondrial signal peptidase

The mitochondrial inner membrane peptidase I (ImpI) is a complex of two homologous but non-identical polypeptide chains, Imp1p and Imp2p with molecular masses of 21 and 19 kDa, respectively (Behrens et al., 1991; Schneider et al., 1994; Nunnari et al., 1993). These proteins are related to the bacterial and ER type I signal peptidases. Both subunits have proteolytic activity but, unlike the signal peptidases of *B. subtilis*, they have distinct, non-overlapping specificities (see below).

Imp1p and Imp2p are associated with the mitochondrial inner membrane and the bulk of each protein is exposed to the intermembrane space as demonstrated by their accessibility to proteinase K in mitoplasts (Schneider et al., 1991; Nunnari et al., 1993). Current models predict that Imp1p and Imp2p have one amino terminal membrane spanning region (Fig. 1A; Dalbey & von Heijne, 1992; van Dijl et al., 1992), although this has not been shown experimentally. In addition, Imp2p may have a carboxyl terminal membrane spanning region, as predicted for the signal peptidase of *R. capsulatus* (Fig. 1D).

Evolutionary concepts

The bacterial, mitochondrial, and ER signal peptidases appear to be members of a new and perhaps previously unrecognized peptidase family (Dalbey & von Heijne, 1992; van Dijl et al., 1992). In a key study, cloning of the gene encoding the *S. cerevisiae* mitochondrial inner membrane peptidase, Imp1p, established that Imp1p is homologous to *E. coli* signal peptidase (Behrens et al., 1991). The aligned amino acid sequences of the signal peptidases of *E. coli*, *S. typhimurium*, *B. subtilis* (SipS), and *S. cerevisiae* Imp1p revealed five regions of similarity denoted A–E. These five regions of similarity are also present in the Sec11-like subunits of the yeast and canine microsomal signal peptidases (van Dijl et al., 1992). In fact, the regions A–E are present in all known type I signal peptidases (Fig. 2).

The first conserved domain, domain A (the predicted membrane anchors in Fig. 3), consists of hydrophobic residues residing in the membrane-spanning regions of all these enzymes, which correspond to the second membrane-spanning region of the *E. coli* signal peptidase (Figs. 1 & 3). Domain B contains a strictly conserved Ser-Met sequence that is probably positioned near the membrane surface on the extracytoplasmic side (Fig. 1). The Ser residue of domain B corresponding to Ser-90 of the *E. coli* signal peptidase, is the only Ser residue which can be aligned in all known

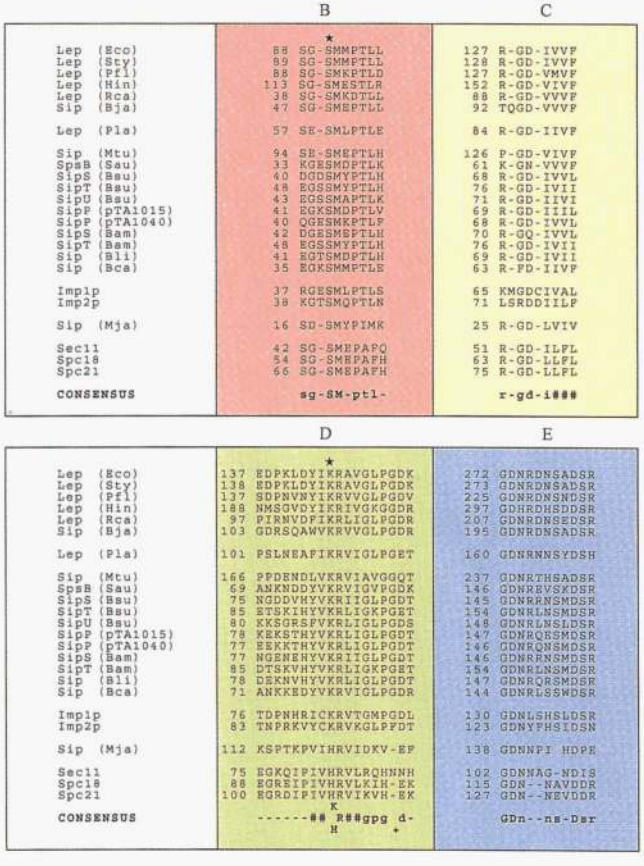

Fig. 2. Conserved domains B–E of signal peptidases. Strictly conserved residues in the consensus sequence are indicated by the upper case letter; conservative substitutions by the lower case letter; and hydrophobic residues by a number sign (#). The serine and lysine/histidine residues that may be involved in catalysis are indicated by an asterisk. Bam, *B. amyloliquefaciens*; Bca, *B. caldolyticus*; Bja, *B. japonicum*; Bli, *B. licheniformis*; Bsu, *B. subtilis*; Eco, *E. coli*; Hin, *H. influenzae*; Imp, Inner membrane protease; Lep, leader peptidase; Mja, *Methanococcus jannaschii*; Mtu, *M. tuberculosis*; Pfl, *P. fluorescens*; Pla, *P. laminosum*; Rca, *R. capsulatus*; Sau, *S. aureus*; Sip, signal peptidase; SPC, signal peptidase complex; Sty, *S. typhimurium*.

signal peptidases. Domain C contains a conserved Gly followed by an Asp.

Domain D contains a Lys-Arg sequence (Lys-145–Arg-146 in *E. coli*) that is strictly conserved in bacterial and mitochondrial enzymes. In contrast, there are no conserved Lys residues in alignments of the ER Sec11p-like sequences; the conserved bacterial Lys aligns with a conserved His in domain D of the ER subunits as well as the putative signal peptidase of the archaeon *M. jannaschii*. The functional significance of this apparent substitution is not known.

Finally, domain E has a conserved tripeptide, Gly-Asp-Asn, a conserved Asp, and a conserved Arg (Fig. 2). The signal peptidase sequences contain no conserved Cys residues. There are two conserved His residues in the alignment of the ER proteins that are not present in the bacterial proteins.

The overall sequence similarities between the bacterial signal peptidases are rather low. In fact, the 18 known enzymes share only 17 identical residues and 16 conservative changes (8.0% identical and similar residues in a consensus length of 415 residues). When the Imp1p and Imp2p subunits of the mitochondrial signal peptidase are included in the comparison with the bacterial signal peptidases only 14 identical residues and nine conservative changes can be detected (5.6% identical and similar residues in a consensus length of 416 residues). Of the 14 identical residues, 12 are located in the conserved regions B (four residues), D (two residues) and E (five residues), and one is the initiator Met. Direct comparison of individual signal peptidases reveals much higher degrees of amino acid similarity. The mitochondrial enzymes are most similar to the *B. licheniformis* signal peptidase with 33% identical and similar residues in a consensus length of 219 residues. Based on this information, it appears likely that the mitochondrial signal peptidases, Imp1p and Imp2p, evolved from a common bacterial ancestor.

The similarity between the Sec11p-like subunits of the ER signal peptidases and the bacterial and mitochondrial (ImpI) signal peptidases is limited to the conserved regions A, B, C, D, and E, and even in these regions only six residues are identical in all known enzymes. Nevertheless, it appears that the bacterial signal peptidases and the Sec11p-like subunits of the ER signal peptidases may share a common ancestor. Alternatively, because of the very limited identities found in global alignments of the amino acid sequences of these two groups of signal peptidases, the sequence relationships in the putative active site regions could be the result of convergent evolution.

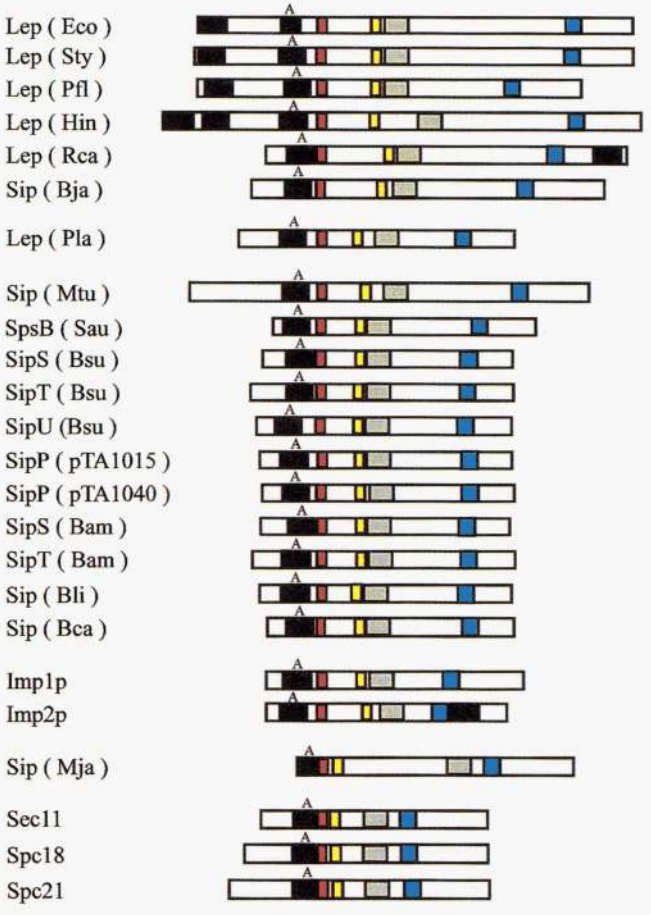

Fig. 3. Chain diagram of bacterial, mitochondrial and ER signal peptidases. The diagram shows the relative locations of the conserved domains A–E (indicated in color: black, predicted membrane anchor including domain A; red, domain B, containing the putative active site serine residue; yellow, domain C; green, domain D; blue, domain E). Acronyms for bacterial names (as in Fig. 2) are indicated in parentheses.

Catalytic mechanism

Since the discovery of signal peptidases, it was proposed that these enzymes are mechanistically novel peptidases as they are resistant to peptidase inhibitors of the classical serine, cysteine, aspartic acid, or metallo classes of peptidases. Site-directed mutagenesis approaches have been used to define the amino acids directly involved in catalysis by the bacterial enzymes. These studies revealed that the conserved Ser in domain B of the family is essential for catalytic activity and may act as the active site nucleophile in signal peptide cleavage. Mutation of this residue to Ala in the *E. coli* (Sung & Dalbey, 1992) and *B. subtilis* (SipS; van Dijl et al., 1995) signal peptidases, as well as the Imp2p subunit of the mitochondrial signal peptidase (Nunnari et al., 1993) yielded inactive enzymes. Substitution of Cys for Ser-90 in the *E. coli* and the corresponding Ser in *B. subtilis* (SipS) signal peptidases produced active peptidases. In the case of *E. coli*, the Cys-90 signal peptidase can be inactivated with N-ethyl maleimide, a cysteine-specific reagent that does not inactivate the wild-type enzyme (Tschantz et al., 1993).

In contrast to classical serine peptidases, the *E. coli* signal peptidase does not employ a His as the proton donor and proton acceptor. Each of the three His residues in this enzyme can be mutated to Ala without any observable effect on enzymatic activity (Sung & Dalbey, 1992; Black et al., 1992). No His residues are conserved in amino acid sequence alignments of the prokaryotic signal peptidase family. Lys-145 in domain D of *E. coli* is present in all bacterial and mitochondrial signal peptidases described to date (Fig. 2) and is a strong candidate to play the role of general base that strips the proton from the nucleophilic Ser during peptide bond cleavage. However, as noted above, this Lys is notably absent in the ER signal peptidases. Substitution of Lys-145 of the *E. coli* signal peptidase by Met (Black, 1993) or by Ala, His, or Asn (Tschantz et al., 1993) abolishes catalytic activity. Similar results were observed with the *B. subtilis* signal peptidase SipS (van Dijl et al., 1995). These experiments established that the critical Lys residue is required for a functional enzyme but do not directly show that this amino acid is involved in the proton transfer at the active site.

Interestingly, the SpsA protein of *S. aureus*, which is very similar to the SipS-like signal peptidases of Gram-positive bacteria, seems to lack the putative catalytic Ser and Lys residues (Cregg et al., 1996). The role of SpsA in the processing of secretory precursor proteins in *S. aureus* has not been investigated yet but it is predicted that this protein has no signal peptidase activity.

At this stage, the signal peptidases of bacteria and mitochondria appear to be unconventional serine peptidases that employ a Lys as a general base. The hydroxyl group of the serine side chain acts as the nucleophile that attacks the carbonyl carbon of the scissile peptide bond of the pre-protein cleavage site. The unprotonated form of the Lys ϵ-amino group serves to activate the hydroxyl group of the Ser. There is a precedent for a mechanism involving a Ser/Lys dyad for a peptidase. The LexA protein, which is involved in the SOS response in *E. coli*, undergoes a self-cleavage reaction that inactivates the protein. This self-processing event employs a Ser as the nucleophile that attacks the peptide bond (Roland & Little, 1990) and a Lys that is deprotonated (Lin & Little, 1989; Little, 1993). Moreover, X-ray crystallographic analysis has shown that the Ser/Lys dyad is at the active site of the UmuD′ protein, a member of the Lex A peptidase family (Peat et al., 1996).

More recently, it has become apparent that there is sequence similarity between the region around the putative catalytic Ser and Lys residues of the *B. subtilis* signal peptidases and the region containing the active site Ser and Lys residues of LexA and other LexA-like peptidases (van Dijl et al., 1995). Also in the LexA-like peptidase family, the putative catalytic Ser and Lys residues are strictly conserved, whereas the other regions differ significantly. Moreover, the self-cleavage sites of the LexA-like peptidases are similar to signal peptidase cleavage sites (Nielsen et al., 1996a, 1996b) with a small amino acid residue at the -1 position and a larger aliphatic amino acid at the -3 position (van Dijl et al., 1995). Taken together, the data suggest that the bacterial signal peptidases and the LexA-like peptidases are mechanistically related and cleave similar substrates.

Substrate specificity

Statistical studies of the sequences surrounding the cleavage sites of signal peptides led to the formulation of the (-3, -1) rule for signal peptidase cleavage of its substrates (von Heijne, 1985). A more recent compilation of signal peptide sequences has confirmed this earlier result and has been used to design a computer program for prediction of signal peptidase cleavage sites in precursor protein sequences (Nielsen et al., 1996a, 1996b). This specificity rule has been confirmed by site-directed mutagenesis on ER (Folz et al., 1988) and bacterial (Fikes et al., 1990; Shen et al., 1991) pre-proteins where a large number of mutations have been made at the -3 and -1 positions in signal peptides. Pre-proteins were cleaved only with Ala, Gly, Ser, Cys, or Pro residues at the -1 position, or with Ala, Gly, Ser, Cys, Thr, Val, Ile, Leu, or Pro residues at the -3 position. Almost any residue could be tolerated at the -2, -4, and -5 positions for cleavage of M13 procoat.

An uncharacterized type I signal peptidase resides in the thylakoid membrane of chloroplasts. The specificity of the thylakoidal signal peptidase, which cleaves the thylakoid transfer sequence from proteins that are transported to the lumen of thylakoids, is similar to that of the *E. coli* signal peptidase. The *E. coli* enzyme can cleave several thylakoidal precursor proteins and the thylakoidal signal peptidase can cleave M13 procoat and the precursor to the yeast alpha factor (Halpin et al., 1989). However, the requirements for processing by the thylakoidal signal peptidase are even more restricted than those for processing by the *E. coli* signal peptidase as shown by site-directed mutagenesis studies of the thylakoidal pre-33 kDa protein; Ala is preferred at the -1 and the -3 positions (Shackleton & Robinson, 1991).

In contrast to the ER and bacterial signal peptidases, the mitochondrial inner membrane peptidase has a more complex specificity requirement. It cleaves mitochondrial inter membrane space proteins (IMS) that are initially synthesized with a bipartite signal sequence that contains a matrix-targeting signal and an IMS sorting signal. After cleavage of the IMS sorting signal, the protein is released into the inter membrane space. Interestingly, the specificity of the Imp1p and Imp2p subunits is not identical: the precursors of the cytochrome oxidase subunit II (pre-COXII) and cytochrome b2 are processed exclusively by Imp1p. In contrast, the precursor form of cytochrome c1 is exclusively processed by Imp2p (Nunnari et al., 1993). While the substrate specificity of Imp2p obeys the (-3,-1) rule, Imp1p only cleaves substrates with Asn at the -1 position. For example, both the Imp1p substrates, pre-COXII and cytochrome b2 contain an Asn at the -1 position (Pratje & Guiard, 1986; Schneider et al., 1991; Behrens et al., 1991).

Substrates and inhibitors

In vitro, the *E. coli* signal peptidase can cleave the precursors of many membrane and secreted proteins to their mature products (Wolfe et al., 1982). These include most bacterial pre-proteins, yeast pre-acid phosphatase, honeybee pre-pro-mellitin, and human pre-hormones such as pre-pro-insulin, pre-growth hormone, pre-interferon and others. In vivo, type I signal peptidase is the principal peptidase responsible for signal peptide cleavage as pre-proteins of a number of exported proteins accumulate at the non-permissive temperature in a temperature-sensitive signal peptidase strain of *E. coli* (Inada et al., 1989), and in conditional-lethal *E. coli* strains in which the signal peptidase gene is controlled by the arabinose promoter (Dalbey & Wickner, 1985) or by the left promoter of phage lambda (van Dijl et al., 1988).

In addition to naturally occurring precursor protein substrates, *E. coli* signal peptidase can process short, synthetic peptide substrates based on the cleavage site region of pre-maltose binding protein (Dev et al., 1990) and M13 procoat (Dierstein & Wickner, 1986; Kuo et al., 1993). However, the peptides are poor substrates for signal peptidase with a k_{cat} of 119 h^{-1} and a K_m of 1 mM for the best substrate (Dev et al., 1990). Nevertheless, with these substrates it was shown that the minimum length for cleavage of peptide substrates was five residues (−3 to +2 of the pre-maltose binding protein), indicating that the recognition sequence for signal peptidase lies between the −3 and +2 positions.

The best substrate currently available for processing by the *E. coli* signal peptidase in vitro is a fusion protein consisting of the signal peptide of the *E. coli* outer membrane protein A (OmpA) attached to the *Staphylococcus aureus* nuclease A protein (Chatterjee et al., 1995). The k_{cat} for signal peptide cleavage at pH 8.0 is 8.73 s^{-1}, two to four orders of magnitude higher than that observed with the best synthetic peptide substrates thus far described. The K_m (16.5 μM) is 50 to 100 times lower, showing that the k_{cat}/K_m is increased by six orders of magnitude. The fact that pre-proteins are better substrates than peptides suggests that there are conformational preferences for a protein substrate which are not fulfilled by synthetic model peptides. The pro-OmpA-nuclease A fusion protein is also an excellent substrate for the chicken microsomal signal peptidase (M. Nusier & M. Lively, unpubl. data).

The *E. coli* and *B. subtilis* signal peptidases are not inhibited by any of the commercially available peptidase inhibitors tested to date. These include o-phenanthroline, ethylenediamine tetraacetic acid, phosphoramidon, 2,6-pyridine dicarboxylic acid, bestatin, tosylamido-2-phenylethyl chloromethyl ketone, 1-chloro-3-tosylamido-7-amino-2-heptanone hydrochloride, phenylmethylsulfonyl fluoride, 4-(amidinophenyl)methanesulfonyl fluoride, N-carbobenzyloxy-L-phenylalanyl chloromethyl ketone, dichloroisocoumarin, elastatinal, aprotinin, chymostatin, leupeptin, antipain dihydrochloride, iodoacetamide, N-ethyl maleimide, L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane, 1,2-epoxy-3-(p nitrophenoxy) propane, pepstatin, and diaxoacetyl-DL-norleucine methyl ester (Zwizinski et al., 1981; Black et al., 1992; Kuo et al., 1993; Vehmaanperä et al., 1993). Similarly, standard peptidase inhibitors are ineffective against the ER signal peptidase (Jackson & Blobel, 1980) as well as the partially purified thylakoidal processing peptidase with one exception: EDTA stimulates processing by the thylakoidal enzyme (Kirwin et al., 1987). In contrast, the mitochondrial Imp1p activity is inhibited by EDTA (Schneider et al., 1991). Most likely, the stimulating and inhibiting effects observed with EDTA do not reflect a direct involvement of a metal ion in catalysis since these enzymes seem to belong to the same family as the bacterial and ER signal peptidases.

While insensitive to classical peptidase inhibitors, the *E. coli* signal peptidase is inhibited by a 23 residue synthetic signal peptide of the M13 coat protein (Wickner et al., 1987) and several pre-proteins that include a proline at the +1 position (Barkocy-Gallagher & Bassford, 1992; Nilsson & von Heijne, 1992). The latter pre-proteins are not cleaved in *E. coli* and act as competitive inhibitors of signal peptidase. Recently, after a long search, two pharmaceutical companies have discovered that certain β-lactam compounds inhibit the *E. coli* signal peptidase (Kuo et al., 1994; Allsop et al., 1995). The best inhibitor reported is a 5S penem derivative, which has an I.C. 50 of 3 μM (Allsop et al., 1995). Although not reported, it is very likely that the 5S-penem compounds react with the active site serine as β-lactams often inhibit serine peptidases (Chabin et al., 1993). Because signal peptidase is required for growth of *E. coli* (Dalbey & Wickner, 1985), such compounds have the potential to be safe antibacterial agents. However, it remains to be seen whether 5S penem derivatives inhibit signal peptidases from pathogenic organisms as well.

Soluble domain, crystals, and future prospects

A water soluble form of the *E. coli* signal peptidase lacking the two transmembrane segments has been produced (Kuo et al., 1993) that is facilitating solution of the first X-ray structure of a bacterial signal peptidase. This mutant (Δ2-75) retains significant activity (k_{cat} down 15-fold) with a pre-protein substrate and accurately cleaves the pro-OmpA-nuclease A substrate in vitro (Tschantz et al., 1995). It is noteworthy that full activity was achieved only in the presence of detergent or phospholipid, suggesting that detergent may be necessary to provide an environment suitable for catalysis even in a form of the protein that lacks its membrane spanning domains. The studies with the truncated fragment of the *E. coli* signal peptidase show conclusively that the active site of the peptidase is located within the periplasmic domain. The truncated signal peptidase forms X-ray diffraction quality crystals in the presence of detergent (Paetzel et al., 1995). The crystals belong to the tetragonal space group ($P4_22_12$) and the best crystals have strong reflections down to 2.3 Å resolution.

Many questions remain unanswered regarding the specificity and the catalytic mechanism of type I signal peptidases. What accounts for the remarkable accuracy of these enzymes in cleaving exported proteins? What aspects of the signal peptidase structure define the signal peptidase cleavage site? What is the catalytic mechanism of signal peptidase and is Lys the general base required for catalysis by the bacterial and mitochondrial enzymes? If so, which amino acid performs this function in the ER signal peptidases lacking this Lys? The availability of a signal peptidase structure will help to answer these questions and to pinpoint the active site and substrate binding sites of signal peptidase. Ultimately, a signal peptidase structure will be useful in the design of new and improved inhibitors which may be of pharmaceutical importance.

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